EP2 and EP4 Receptors Differentially Mediate MAPK Pathways Underlying Anabolic Actions of Prostaglandin E_2 on Bone Formation in Rat Calvaria Cell Cultures

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Abstract

Of the four prostaglandin (PG) E receptor subtypes (EP1-EP4), EP2 and EP4 have been proposed to mediate the anabolic action of PGE_2 on bone formation and some comparative evaluation studies of EPs on bone formation have been done, but their relative impacts on bone formation including downstream MAPK pathways remain unresolved. To dissect this issue, we systematically assessed the roles of EPs in the rat calvaria (RC) cell culture model by using four selective EP agonist (EPA)s. Consistent with relative expression levels of the respective receptors, multiple phenotypic traits of bone formation *in vitro*, including proliferation of nodule-associated cells, osteoblast marker expression and mineralized nodule formation were upregulated not only by PGE_2 but equally by EP2A and EP4A, but not by EP1A and EP3A. EP2A and EP4A were effective when cells were treated chronically or pulse-treated during nascent nodule formation. EP2A and EP4A equally stimulated the endogenous PGE_2 production, while EP2A caused a greater increase in cAMP production and c-fos gene expression compared to EP4A. EP2A and EP4A activated predominantly p38 MAPK and ERK respectively, while c-Jun N-terminal kinase (JNK) was equally activated by both agonists. SB203580 (p38 MAPK inhibitor) blocked the PGE_2 effect on mineralized nodule formation, while U0126 (ERK inhibitor) and dicumarol (JNK inhibitor) were less effective. PGE_2-dependent phosphorylation of the MAPKs was affected not only by protein kinase (PK)A and PKC inhibitors but also by adenylate cyclase and PKC activators. Co-treatment of RC cells with EP2A or EP4A and bone morphogenetic protein (BMP)2, whose effects on bone nodule formation is known to be, in part, mediated through the PKA and p38 MAPK pathways, resulted in an additive effect on mineralized nodule formation. Further, PGE_2, EP2A and EP4A did not increase BMP2/4 mRNA levels in RC cells, and

EP2-induced phosphorylation of p38 MAPK was not eliminated by Noggin. These results suggest that, in the RC cell model, the anabolic actions of PGE_2 on mineralized nodule formation are mediated at least in part by activation of the EP2 and EP4 receptor subtype-specific MAPK pathways, independently of BMP signaling, in cells associated with nascent bone nodules.

Key word: selective EP agonists, rat calvaria cells, osteoblastogenesis, MAPKs

Introduction

It is well established that prostaglandin (PG) E_2 increases bone mass and strength when administered systemically or locally to the skeleton by acting on the growth and differentiation of osteoblastic cells [1]. Over the last decade, the molecular mechanisms underlying PGE_2 actions on bone formation are becoming apparent; for example, PGE_2 alters expression of molecules which are crucial for osteoblast development and function, including runt-related transcription factor 2 (Runx2) [2] and transforming growth factor (TGF)-β type III receptor [3]. However, how the signaling output from PGE receptor (EP) subtypes contributes to bone formation is not fully resolved. Four specific G protein-coupled EP subtypes (EP1-EP4) have been identified and are known to be differentially expressed across tissue types [4]. It is also known that EP1 couples to Ca^{2+} mobilization, and EP2 and EP4 activate, whereas EP3 inhibits, adenylate cyclase. Given such a multiplicity of biological responses, it is not surprising that PGE_2 is involved in a number of physiological and pathophysiological events [5].

After each of the four EP receptor subtypes-lacking mice were generated, which receptor plays a role in bone metabolism has been investigated. A study using EP1 and EP2 receptor knockout mice showed that EP2 receptor has a major influence on the biomechanical properties of bone rather than EP1 receptor [6], and the studies using EP4 knockout mice [7] and cell culture derived from those [8] demonstrated that the lack of EP4 receptor had no influence of PGE_2-induced bone formation. Recently, new agonists with high affinity and selectivity for each EP receptor subtype (EP1A-EP4A) have been described [5, 9]. A local

injection of CP-533,536, an EP2-selective agonist, stimulates bone formation in canines [10] and rats [11] fracture and/or normal models, suggesting that EP2plays a role in PGE_2-dependent bone formation. On the other hand, the studies conducted daily injection of ONO-4891 [12] and CP-734,432 [13], both EP4-selective agonists, in rats show that EP4 agonists have a potential of the prevention and treatment of osteoporosis.

PGE_2 is involved in activation of mitogen-activated protein kinase (MAPK)s [14], and which MAPKs are linked to EP receptor subtypes has been extensively investigated in non-skeletal cells, such as colorectal tumors [15]. For example, EP4 but not EP2 signaling leads to phosphorylation of the ERKs through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway in HEK 293 cells ectopically expressing EP2 and EP4 [16]. What about bone? Limited information is available on the question of how EPs activate MAPKs for bone formation. PGE_2 stimulates fibronectin through the EP1/phospholipase C/protein kinase (PK)C/c-Src signaling pathway in rat osteoblasts [17]. The PKC pathway is also involved in PGE_2-induced cell proliferation via ERK in the MC3T3-E1 osteoblastic cell line [14].

The diverse effects of PGE_2 on bone *in vivo* and osteoblastic cells *in vitro* suggest that unambiguous understanding of the molecular mediators of the anabolic action of PGE_2 on bone formation is still lacking. In addition, in spite of the marked effects of PGE_2 on bone formation, its receptor subtype-dependent multiple actions cause severe side effects including diarrhea, lethargy, and flushing [18], making it an unacceptable therapeutic option in humans. Thus, simple but systematic approaches that reflect *in vivo* phenomena are still

required to address the molecular pathways by which EP receptor subtypes act on osteoblast development. To this end, we have compared the effects of EP1A-EP4A in well-established rat bone formation models *in vitro* and found that MAPK pathways active predominately in nascent nodule-forming cells, which may contribute to PGE_2 actions on bone formation in an EP2 and EP4 receptor subtype-specific dependent manner.

Materials and Methods

Reagents

Selective EP agonists (EP1A, ONO-DI-004; EP2A, ONO-AEI-259; EP3A, ONO-AE-248; EP4A, ONO-AE1-437) were gifts from Ono Pharmaceutical Co. (Osaka, Japan) [19]. Both recombinant human bone morphogenetic protein 2 (rBMP2) and Noggin (rNoggin), an antagonist of BMPs, were purchased from PeproTech (Rocky Hill, NJ). H89, a PKA inhibitor, and Go6850, a PKC inhibitor, were obtained from D. Western Therapeutics Institute (Nagoya, Japan) and Calbiochem (Darmstadt, Germany), respectively. Forskolin (an adenylate cyclase activator), phorbol 12-myristate 13-acetate (PMA, an activator of PKC), PGE_2, MAPK inhibitors (for p38 MAPK, SB203580; for c-Jun N-terminal kinase (JNK), dicumarol; for ERK, U0126) and all other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St Louis, MO). Stock solutions were prepared in dimethylslufoxide and diluted with medium (1,000 times or more) before use.

Animals

Animal use and procedures were approved by the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University and the University of Toronto Animal Care Committee. Rats were euthanized by cervical dislocation or deep anesthesia.

Tissue and cell preparation and immunohistochemistry

Twenty-one-day-old rat fetal (embryonic day 21 (E21)) and newborn Wistar rat (day 8) parietal bones were fixed in 4% paraformaldehyde and embedded in paraffin using standard protocols. Cells grown on coverslips at day 7 (nascent nodule formation stage, see below)

were fixed and stored in acetone at –20 °C until use. Coronal sections (4.5 μm in thickness) or cultured cells were treated with antibodies against EP2, EP4 or Runx2 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by incubation with Cy™2- or Cy™3-conjugated secondary antibody (1:400; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h. Each incubation step was followed by two washes with PBS (5 min each). As negative control, normal goat or rabbit IgG (Vector Lab, Burlingame, CA) or vehicle alone replaced primary antibodies. Neighboring sections were stained with hematoxylin and eosin.

Cell cultures

Rat calvaria (RC) cells were routinely obtained from fetuses (Wistar, E21) as described [20]. Briefly, calvariae were minced and digested with collagenase (type I) for 10, 20, 30, 50 and 70 min at 37 °C. Cells retrieved from the last four of five digestion fractions were separately grown in αMEM containing 10% fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel; Cansera, Etobicoke, Canada) and antibiotics. After 24 h, cells were pooled and grown in multi-well plates or 35-mm dishes (0.3×10^4 cells/cm^2) in the same medium supplemented additionally with 50 μg/ml of ascorbic acid. Cells were treated with or without reagents in regular or serum-deprived conditions (see below), as specified. To induce matrix mineralization, 10 mM β-glycerophosphate (βGP) was added into cultures for 2 days before culture termination. Medium was changed every 2-3 days, and cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

5-bromo-2'-deoxyuridine (BrdU)-labeling index

Cells at day 7 were serum-deprived (0.1% FCS) for 24 h, and then treated with or without EP agonists or PGE_2 for 24 h, followed by pulse treatment with BrdU (10 μM) for 2 h before culture termination. After fixation in 70% ethanol for 30 min and air drying, cells were permeabilized with 2 M HCl for 5 min. BrdU-labeled cells were detected immunohistochemically by using anti-BrdU monoclonal antibody (1:1,000) and horseradish peroxidase-labeled goat anti-mouse IgG (1:200, Vector Lab) with the avidin-biotin complex (ABC) system (Vector Lab). Each step was followed by two washes (5 min each) with PBS.

Colony formation assay

To obtain colony-forming unit-osteoblasts (CFU-O), cells were plated at very low density (≤200 cells/100-mm dishes) with 10 nM dexamethasone, a potent stimulator of osteoblast development in this model (14). CFU-fibroblastic/primitive progenitor cells (CFU-F) were also obtained; colony types were identified by RT-PCR for markers as below. CFU-alkaline phosphatase (CFU-ALP) was obtained by plating at a low density (≤200 cells/well) in 96-well plates with or without EP agonist treatment. Wells occupied by ALP-positive (ALP^+) cells (ALP staining, see below) were counted in some experiments.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's directions. cDNA was synthesized from ≤2 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) at 50 °C for 40 min. Primer sets for rat

osteoblast markers and L32 as internal control were described elsewhere [21]; osteopontin (OPN), 5'-AGA GGA GAA GGC GCA TTA CA-3' and 5'-GCA ACT GGG ATG ACC TTG AT-3'; ALP, 5'-GAT AGG CGA TGT CCT TGC AG-3' and 5'-TTA AGG GCC AGC TAC ACC AC-3'; bone sialoprotein (BSP), 5'-CGC CTA CTT TTA TCC TCC TCT G-3' and 5'-CTG ACC CTC GTA GCC TTC ATA G-3'; osteocalcin (OCN), 5'-AAC GGT GGT GCC ATA GAT GC-3' and 5'-AGG ACC CTC TCT CTG CTC AC-3'; Runx2, 5'-CTT CAT TCG CCT CAC AAA C-3' and 5'-CAC GTC GCT CAT CTT GCC GG-3'; ribosomal protein L32, 5'-CAT GGC TGC CCT TCG GCC TC-3' and 5'-CAT TCT CTT CGC TGC GTA GCC-3'. Primer sets for rat proliferation-related markers and BMPs were designed using Primer Picking (Primer 3) as follows: c-fos, 5'-AGA ATC CGA AGG GAA AGG AA-3' and 5'-ATG ATG CCG GAA ACA AGA AG-3'; cyclin D1, 5'-TCC CGC CAG CAG CAA GAC AC-3' and 5'-TGA GCT TGT TCA CCA GAA GC-3'; BMP2, 5'-TGA ACA CAG CTG GTC TCA GG-3' and 5'-ACC CCA CAT CAC TGA AGT CC-3'; BMP4, 5'-CGT TAC CTC AAG GGA GTG GA' and 5'-AGT CCA CGT AGA GCG AAT GG-3'. Primer sets for rat EP receptors were: EP1, 5'-TAG TGG ATG AGG CAA CAA CG-3' and 5'-GCT GTG GTT GAA GTG ATG GA-3'; EP2, 5'-GGC TTC TTA TTC GAG AAA CCA GAC CTA GTG GC-3' and 5'-AGG TCC CAC TTT TCC TTT CGG GAA GAG GTT TCA TC-3'; EP3, 5'-TGT CTA GGC TTG CTG GCT CT-3' and 5'-TGG AAG CAT AGT TGG TGT GG-3'; EP4, 5'-CCT TCT CTT ACA TGT TAC GCG GGC TTC AG-3' and 5'-TGC TTT CAG TTA GGT CTG GCA GGT ATA GGA GG-3'. Real-time RT-PCR was carried out by using the Light Cycler system (Light Cycler™ DNA Master SYBR®Green I; Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. For

semiquantitative assessment of expression levels, each PCR reaction was realized for different cycles from 16 to 40 cycles, based on points at mid-log phase of the cycles as described elsewhere [22]. PCR products were then size-fractionated on 1% ethidium bromide/agarose gels.

Western blotting

Cells in serum-deprived conditions (0.1% FCS) for a day were treated with reagents as described in specific experiments. Cells were then washed with cold PBS and lysed in 100 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan) and Complete Protease Inhibitor Cocktail (Roche Diagnostics) in 50 mM Tris-HCl (pH7.5). After centrifugation, aliquots of the cell lysates (≤20 μg protein/lane) were subjected to SDS-PAGE (15% gels) and electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated with 0.5% casein in TTBS (20 mM Tris-HCl, 137 mM NaCl, pH 7.6 containing 0.1% Tween 20) for 1 h and probed with antibodies against phosphorylated p38 MAPK, JNK, and ERK1/2 (1:1,000; Santa Cruz biotechnology) at 4 °C overnight. The membranes were then incubated with HRP-conjugated secondary antibody (1:2,000, Santa Cruz Biotechnology) for 1 h, and subjected to chemiluminescent detection (Lumi-Light^{PLUS}, Roche Diagnostics). Each step was followed by three washes (5 min each) with TTBS. The membranes were then reprobed with corresponding antibodies against non-phosphorylated MAPKs (1:1,000, Santa Cruz Biotechnology).

ALP/von Kossa staining

Cultures were fixed in neutral buffered formalin for 15 min. After washing, cultures were incubated with AS MX-phosphate/blue or red LB in 0.1 M Tris-HCl (pH 8.3). To confirm mineralized nodules, cultures were further treated with 2.5% silver nitrate solution.

Medium PGE_2 measurement

Cells cultured in serum-deprived conditions for a day (day 7) were pretreated in the presence or absence of 1 μM NS-398, an inhibitor of cyclooxyganase (COX)-2 for 3 h and then treated with or without EP agonists or PGE_2 for 4 h. To avoid the interference of the additive reagents, the cells were washed twice with serum-free medium. After an additional incubation for 24 h, the culture medium was collected for PGE_2 measurement (Prostaglandin E_2 EIA Kit, Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's protocols.

Intracellular cAMP measurement

Cells at day 7 in serum-deprived conditions (see above) were pretreated with 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) for 20 min, followed by treatment with or without EP agonists, PGE_2 (≤1 μM each) or forskolin (10 μM) for 15 min. After washing with ice-cold PBS, cell lysates were prepared and subjected to cAMP Biotrak Enzymeimmunoassay System (GE Healthcare Biosciences, Little Chalfont, UK), according to the manufacturer's instructions.

Statistical analysis

Data from at least triplicate samples are expressed as the mean ± SD, and two independent experiments were performed at a minimum. Statistical differences were evaluated by analysis of variance (ANOVA) and post hoc Student's t-test.

Results

Both EP2A and EP4A act on nascent nodule-forming cells in RC cell cultures

To assess which EP receptor subtypes are involved in PGE_2-dependent mineralized nodule formation, we chronically treated with PGE_2 or each of four EP agonists in RC cell cultures. Similarly to PGE_2, EP2A and EP4A but not EP1A and EP3A dose-dependently increased the number of mineralized nodules (Figure 1A-C) and expression of osteoblast marker mRNAs (ALP and OCN) (Figure 1D). These effects are comparable to that of rBMP2 as positive control (Figure 1C). To determine whether agonist dependency is correlated with corresponding receptor expression, we next asked whether EP receptors are expressed in single cell-derived colonies, CFU-F and CFU-O (Figure 1E). RT-PCR showed that EP2 and EP4 mRNAs are expressed not only in CFU-O but also in CFU-F. EP1 mRNA was only faintly detected (at over 40 cycles of PCR) in both colony types, whereas EP3 mRNA was not detected at all.

Previous studies indicated that exposure of either RC or rat bone marrow stromal cells to PGE_2 at relatively early time in culture increases bone nodule formation [23, 24]. To identify more precisely at which developmental stages are the target cells for EP2A and EP4A, we categorized RC cultures into four typical developmental time windows: *i.e.*, proliferating progenitor cells (day 2-5), early differentiating precursor cells associated with nascent nodules (day 5-8), preosteoblasts and immature osteoblasts (day 8-11) and maturing osteoblasts (day 11-14) (Figure 2A), and pulse-treated with PGE_2, EP2A or EP4A in the different time windows. PGE_2 and both agonists elicited the largest increase in the number of mineralized nodules formed when cultures were treated at times corresponding to early

differentiation-nascent nodule formation stages (Figure 2B); pulse treatment during this window elicited effects as large as chronic exposure to PGE_2. Concomitantly, we found that PGE_2, EP2A or EP4A increased BrdU-labeling dominantly in cells associated with nascent nodules (Figure 2C, D). Furthermore, PGE_2, EP2A and EP4A all increased the abundance of CFU-ALP (Figure 2E). In contrast, treatment with rBMP2 during the developmental time window in which PGE_2 effects were maximal effects (early differentiation-nascent nodule formation) elicited no significant effect on mineralized nodule formation (Figure 2F).

To determine whether the developmental time-specific effects of EP2A and EP4A correlate with expression levels of EP receptor subtypes, we compared EP2 and EP4 mRNA levels with osteoblast markers over the developmental sequence; osteoblast markers were ALP, a relatively early marker of osteoblast differentiation; OPN, which peaks during nodule development stages; and BSP and OCN, late markers of osteoblast differentiation (Figure 3A). Consistent with the effects elicited by pulse treatment with agonists, both EP2 and EP4 mRNAs were highly expressed at day 7, and were downregulated before BSP and OCN mRNA expression peaked (by day 10). Further, we established the spatial localization of EP2 and EP4 in both RC cell cultures and E21 fetal rat parietal bones by immunofluorescence staining. In day 7 RC cell cultures, EP2 and EP4 were co-localized to Runx2-positive cells in nascent nodules (Figure 3B). Immunohistochemistry on E21 fetal parietal bones, the same embryonic day used to isolate RC cells for cultures, demonstrated that EP2 and EP4 were most intensely stained in osteoblasts and their surrounding preosteoblastic cells (Figure 3C). Taken together, these results suggest that the expression patterns of EP2 and EP4 receptor subtypes are closely correlated with an apparent site of

action of PGE_2 on nodule formation.

EP2A and EP4A elicit overlapping but distinct osteogenic and signaling pathway activities in RC cell cultures and are independent of BMP2

Because both EP2 and EP4 are known to share second messenger signaling [25], it is not surprising that both agonists show similar effects on the osteoblastic phenotypes in RC cell cultures. However, when we compared the response of cAMP to EP2A and EP4A stimuli in RC cells at day 7, EP2A exhibited a robust effect with dose dependency, in comparison to EP4A or PGE_2 (Figure 4A), suggesting that PGE_2 may activate the PKA pathway mostly via EP2 in this model. We further assessed the effect of EP2A and EP4A on the expression of the c-fos protooncogene [26] and cyclin D1 [27] which are upregulated by cAMP-dependent pathways in the mouse osteoblastic MC3T3-E1 and chondrocytic MMA2 cell lines, respectively. Similarly to the effects on cAMP production, EP2A elicited a significant larger effect than EP4A on c-fos and cyclinD1 (Figure 4B).

Downregulation of PKA inhibitor G (PKIG) expression in human and mouse stromal cells is necessary for BMP2-induced osteogenic differentiation [28], while increased cAMP levels potentiate BMP4-dependent osteo/chondrogenesis in C3H10T1/2, STC2 and MC3T3-E1 cell lines [29]. Thus, although it is unknown how BMP contributes to these processes (at least Smads do not mediate PKIG expression [28]), the PKA pathway appears to be involved in BMP-dependent osteoblast differentiation. Therefore, we next determined whether EP2A and EP4A differentially regulate BMP2 and BMP4 mRNA expression in RC cells; amongst conditions tested, BMP2 mRNA levels were decreased by both EP2A and

EP4A while BMP4 mRNA levels were not changed (Figure 4C).

An alternative possibility for the differences seen in some parameters in EP2A- versus EP4A-treated RC cell cultures is that EP2A and EP4A may differentially modulate endogenous production of PGE_2 [30]. We therefore measured PGE_2 production in RC cells at day 7 in the presence or absence of PGE_2, EP2A or EP4A. All three agonists increased PGE_2 levels in the medium (at least in the absence of NS-398), but there was no significant difference between EP2A and EP4A treatments (Figure 4D). The effect of EP2A and EP4A was higher than that of PGE_2 (in the presence of NS-398) (Figure 4D). Notably, co-treatment of RC cells with EP2A and EP4A had an additive effect on mineralized nodule formation (Figure 4E). Likewise, either a combination of EP2A and rBMP2 or a combination of EP4A and rBMP2 was additive (Figure 4E). These results suggest that there may be both common and distinct pathways underlying the EP2 and EP4 actions on mineralized nodule formation and that the signaling pathways elicited by PGE_2 and BMP2 and underlying effects on mineralized nodule formation, while sharing some similarities (e.g., induction of PKA) are not identical.

PGE_2 promotes osteoblastogenesis via summation of its multiple effects on MAPK pathways involved in PKA and PKC signaling in RC cells

Because locally released PGE_2, acting via PKC-dependent ERK activation, was found recently to be involved in TGF-β-induced proliferation of MC3T3-E1 cells [14], we assessed whether EP2A and EP4A differentially activate MAPK. When RC cells were

treated at day 7, EP2A and EP4A activated predominately p38 MAPK and ERK respectively, and JNK equally (Figure 5A). BMP2 activates the p38 MAPK pathway, an effect blocked by Noggin, in the human osteosarcoma MG63 and fetal osteoblastic cell lines [31], however rNoggin did not inhibit the EP2A-dependent phosphorylation of p38 MAPK in RC cells (Figure 5B). To further address whether MAPKs are involved in PGE_2 actions in our model, we pretreated RC cells at day 7 with MAPK inhibitors and assessed the effect of PGE_2 on mineralized nodule formation (Figure 5C). Consistent with a report that p38 MAPK plays a positive role in induction of one of the master transcription factors for osteoblast development, osterix, by BMPs in mouse calvaria osteoblastic cells [32], the p38 MAPK inhibitor SB203580 blocked mineralized nodule formation in the presence or absence of PGE_2, whereas the JNK inhibitor dicumarol and the ERK inhibitor U0126 only partially blocked the PGE_2 effect. To address further how the MAPK pathway is involved in PGE_2-dependent osteogenesis, we tested rapid (30 min) responses of RC cells at day 7 to PGE_2, *i.e.*, transcriptional activation of c-fos and Runx2, the latter another master gene for osteoblastogenesis [33] (Figure 5D); similarly to c-fos in MC3T3-E1 cells [26], Runx2 activation is mediated by a cAMP-dependent pathway in the UMR106-01 rat osteosarcoma cell line [34]. All three inhibitors examined here blocked the induction of Runx2 mRNA by PGE_2, while c-fos mRNA expression was downregulated only by SB203580. Taken together, these results suggest that each MAPK cascade may be differentially involved in PGE_2-dependent osteogenic activities in RC cell cultures. To further confirm these cascades, we pretreated RC cells at day 7 with H89 or Go6850 and evaluated the effects of PGE_2 on the phosphorylation of MAPKs (Figure 5E). H89 blocked PGE_2-dependent phosphorylation of p38 MAPK and JNK, while Go6850 inhibited only that of ERK. Similarly, we found that

p38 MAPK and ERK were activated by forskolin and PMA, an activator of PKC, respectively (Figure 5F). There results suggest that not only PKA but also PKC is involved in PGE_2-dependent activation of MAPKs in RC cells.

Discussion

We systematically evaluated PGE_2 actions on bone formation and the associated MAPK signaling pathways involved in well-established fetal/newborn RC models by using selective EPAs, which already known . We found, first, that amongst the four EP receptor subtypes, EP2 and EP4 mediate PGE_2 effects on mineralized nodule formation. Second, PGE_2 acts primarily to stimulate proliferation and differentiation of precursor cells associated with newly forming bone nodules. Third, these biological activities were mediated, at least in part, by EP2 (primarily p38 MAPK)- and EP4 (mainly ERK)-dependent MAPK cascades. Finally, these signaling cascades did not entirely overlap with and are distinct from those of BMP2. Taken together, our data suggest that the anabolic effect of PGE_2 on bone formation may be mediated through multiple EP2- and EP4-MAPK signaling pathways.

Early studies exploring EP receptor subtype expression in various osteoblastic cell models (cells from rat calvariae and/or tibiae and MC3T3-E1 cells) [8, 35, 36] gave inconsistent results. All four EPs were subsequently detected in primary osteoblastic cells derived from newborn mouse [9] and fetal rat calvariae [37]. However, because primary cells from calvariae comprise multiple cell types including non-osteoblastic/fibroblastic cells, we further discriminated which cells express which receptor subtype by analyzing single cell-derived colonies, and found no detectable EP3, very low expression of EP1 and robust expression of EP2 and EP4. In keeping with this, EP1A and EP3A had no detectable effects on mineralized nodule formation in our model, suggesting that - although EP1 [24, 37] and EP3 [24] signaling has been measured - PGE_2 response may be mediated mainly through

EP2 and EP4 in the RC model. However, given our data on the relative potencies of PGE_2 versus EP2A and/or EP4A on some parameters (for example, see Figure 4A and D), it remains possible that PGE_2 may stimulate not only EP2/EP4 but also EP1, the latter antagonizing the effects of EP2A and/or EP4A.

Growing evidence suggests that not only EP2 but also EP4 is involved in osteoblast development [10-12, 24, 38-41]. However, there are discrepant data on EP2, *i.e*., data from selective agonists (CP-533,536) in canines [10] and rats [11] vs. PGE_2 treatment of *ep2*$^{-/-}$ versus *ep4*$^{-/-}$ mice [12]. Direct comparative analyses of either EP2A and EP4A or EP2A and PGE_2 treatment on osteogenic response in fetal rat long bone organ cultures [36], mouse calvaria cells and/or bone marrow cells [37, 38] have been reported previously. Amongst these reports, the former based on a proline uptake assay in long bones [36] is consistent with our data, but the latter two cell culture reports are discrepant on multiple levels. The basis of the discrepancies is not entirely clear, but it is worth noting that the two earlier cell culture models were analyzed with chronic exposure to NS-398 to inhibit COX-2 activity, and so the answer may lie in differences in endogenous PGE_2 production. It is known that disruption of the *cox-2* gene in mice promotes cell proliferation and inhibits mineralized nodule formation in calvaria cell and bone marrow cultures, and PGE_2 rescues mineralized nodule formation in *cox-2*$^{-/-}$ bone marrow cultures [42]. Thus, PGE_2 production in osteoblastic cells is necessary for mineralized nodule formation in which the PKA pathway is involved [36]. However, the results from the mouse calvaria cell model treated in the presence of NS-398 indicates that the effects of EP2A on ALP activity and OCN mRNA levels are less than those of EP4A, which is opposite to their activities on cAMP production

[26, 36]. Interestingly, the endogenous PGE_2 production elicited by EP2A and EP4A is larger than that by PGE_2 only when the mouse cells were pretreated with NS-398; thus, EP2A and EP4A differentially increase COX-2 transcriptional activity in the presence (EP2A<EP4A) or absence (EP4A>EP2A) of NS-398 in mouse calvaria cells [26]. In our RC model, EP2A and EP4A induce approximately equally PGE_2 production with or without NS-398 co-treatment and both EP2 and EP4 equally and directly contribute to osteoblast development. While additional experiments will be needed to clarify these issues further, the data suggest that EP4 may also mediate mineralized nodule formation independently of the PKA pathway (see below). This view is supported by the fact that, in the RC model, low concentrations (100 nM or less) of selective EP2 and EP4 agonists with high affinity and selectivity [5, 9] were effective, reducing the possibility of crossreactivity amongst EPs. Second, the RC cell model, as underscored by the quantitative CFU assay, is relatively rich in osteoprogenitor and osteoblastic cells versus other lineages, reducing confounding effects of PGE_2 on non-osteoblastic cells (for example, [43, 44]). Third, the RC cell model undergoes sequential stages of osteoblast development and bone formation that are well-characterized at the molecular and morphological levels [45], allowing us to address the stage-specificity of each agonist and dissect out the complex and overlapping multiple effects of PGE_2. As already raised, both positive and negative effects of PGE_2 on proliferation/differentiation have been described in different osteoblastic cell models [46] but only a few studies have been directed at trying to separate effects of PGE_2 on more or less differentiated osteoblast precursors. For example, formation of mineralized nodules was increased when post-confluent RC cell cultures were treated with PGE_2 [23]. CFU characterized by slowly proliferating ALP-negative cells were increased in limiting dilution

analysis of PGE_2-treated RC cells [47]. Our data indicate that at least one major target cell of PGE_2 is the osteogenic precursor that can further proliferate and differentiate [45].

The molecular mechanisms underlying PGE_2 effects have been elucidated in several cell types including cancer cells [18]. However, until recently, the signaling pathway(s) by which PGE_2 directly influences osteoblast development has remained elusive. Although almost all parameters measured here showed that the efficacy of EP2A was similar or a little higher than that of EP4A, the EP2 receptor elicited much more cAMP production than EP4, as discussed above (see also [30]). Because EP2A and EP4A show similar physicochemical properties [5, 9], one possibility is that the differences in efficacy may be attributable to differences in EP2 and EP4 receptor expression, such as relative level and distribution [48, 49]. Additionally, our data strengthen the linkage of PGE_2 to MAPKs as recently described in MC3T3-E1 cells [14]. Insulin-like growth factor-I-mediated osterix expression in human mesenchymal stem cells required all three MAPK pathways (p38 MAPK, JNK and ERK) and PKD signaling, whereas BMP2 requires p38 MAPK during osteoblast linage progression [50]. BMP [51, 52], PKC [53], and PKA [54] pathways are all reported independently to regulate Runx2 gene expression and/or its transactivation function. Consistent with previous data supporting a role for p38 MAPK in regulation of osteoblast differentiation [32], our results show that p38 MAPK has a dominant role in mineralized nodule formation, regardless of the presence or absence of PGE_2, but BMP2 production and its downstream effects on p38 MAPK cannot account for the PGE_2-dependent p38 MAPK signaling effects on osteoblast differentiation in RC cells (Figures 4C and 5B). While chronic treatment with dicumarol and U0126 only partially blocked the PGE_2 effect on

mineralized nodule formation, pulse treatment with these agents inhibited Runx2 expression more markedly than did SB203580. This discrepancy may be due to involvement of other osteoblastogenesis regulators during the chronic treatment. Unexpected upregulation of c-fos expression by dicumarol combined with or without (data not shown) PGE_2 may suggest that the JNK pathway negatively regulates cell proliferation in osteoblasts as it does in liver myofibroblasts [55]. Our data with PKA/PKC activators/inhibitors also suggest that EP4 may activate the PKC pathway which contributes to ERK activation in RC cells, as it does in HEK-293 EBNA cells expressing EP4 [16]. Taken together, our results support the important conclusion that the activation of MAPKs by PGE_2 may be differentially regulated in a receptor subtype-dependent manner, which is the first evidence for a difference in EP2- and EP4-dependent signaling in osteoblasts.

In summary, we systematically characterized PGE_2 actions on bone formation in the RC cell model. EP2 acts principally through the cAMP-p38 MAPK-c-fos/Runx2 pathway, while EP4 may also stimulate ERK, possibly via PKC, and is involved in Runx2 expression. JNK appears to be activated equally by both EP2 and EP4, which may also contribute to regulation of Runx2 expression. These MAPK pathways mediate the osteoblastogenic activities of PGE_2, with a resultant increase in bone formation.

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Figure Legends

Figure 1. EP2A and EP4A stimulate osteoblastogenesis in RC cell cultures.

Cells were chronically treated with or without EPAs, PGE_2 (≤100 nM each) or rBMP2 (≤10 ng/ml) for 14 days and then either stained for ALP/von Kossa (A-C) or used for total RNA isolation (D). (A) Representative macrographs of cultures. (B) and (C) The number of mineralized nodules. (D) Real-time RT-PCR analysis of ALP and OCN. (E) RT-PCR analysis of EP1-EP4. CFU-O and CFU-F were identified on the basis of osteoblast marker (ALP, BSP and OCN) expression. *p<0.05 and **p<0.01, compared to control (–). $^{\#}p$<0.05, compared to EP2A. N.S., no significant difference.

Figure 2. The stage-specific effects of EP2A and EP4A in RC cell cultures.

(A) Schematic of osteoblast development in RC cell cultures (See the Results). (B) The number of mineralized nodules. Cells were pulse-treated with EP2A, EP4A or PGE_2, or chronically treated with PGE_2 (100 nM each) in the indicated time windows. (C) Representative micrographs of BrdU-immunoreactive cells. Cells at day 7 were treated with or without EP2A, EP4A or PGE_2 (data not shown) (100 nM each) for 24 h under serum-deprived conditions. The lower right-hand panel shows the central area of the EP2A panel (upper right-hand panel) at higher magnification. Arrows indicate nodules. Cells were counterstained with hematoxylin. (D) The number of $BrdU^+$ cells as shown in (C). (E) The relative abundance of CFU-ALP. To determine CFU-ALP, cells were seeded at a low density, chronically treated with or without EP2A, EP4A or PGE_2 (100 nM each) for 14 days and then stained for ALP. (F) The number of mineralized nodules. Cells were

pulse-treated with 10 ng/ml rBMP2 in the indicated time windows. *p<0.05 and **p<0.01, compared to control (–). $^{\#\#}p$<0.01, compared to EP2A at the same stage. N.S., no significant difference.

Figure 3. The expression profiles of EP receptor subtypes in RC cells and fetal rat parietal bones.

(A) Real-time RT-PCR analysis of EP2, EP4 and osteoblast markers (ALP, OPN, BSP and OCN) during osteoblast development in RC cell cultures. (B) Immunofluorescence staining. Runx2 was used to identify nascent nodules. Overlay images of immunofluorescence staining of EP2 or EP4 (the upper left- or right-hand panels, respectively) and Runx2 (the middle panels). NC, negative control. (C) Immunofluorescence staining of EP2 and EP4 in fetal rat (E21) parietal bones. Lower left-hand and central panels are higher magnifications corresponding to the sagittal sutures (circles) in each of the upper panels. H&E, hematoxylin and eosin staining. NC, negative control. Scale bars, 20 μm.

Figure 4. EP2A and EP4A elicit distinct signaling pathways essential to osteoblastogenesis at the nascent nodule formation stage in RC cell cultures.

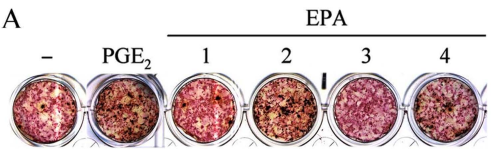
(A) Determination of cAMP production. Cells at day 7 were pretreated with 0.5 μM IBMX and treated with or without EP2A, EP4A, PGE_2 (≤1 μM each) or 10 μM forskolin (FSK) for 15 min under serum-deprived conditions. (B) Real-time RT-PCR analysis of c-fos and cyclin D1. Cells at day 7 were treated with or without EP2A, EP4A, PGE_2 (100 nM each) or 10 μM FSK for 36 h under serum-deprived conditions. (C) Real-time RT-PCR analysis of

BMP2 and BMP4. Cells at day 7 were treated with or without EP2A, EP4A and PGE_2 (100 nM each) for 24 h. (D) Determination of secreted PGE_2. Cells at day 7 were pretreated with or without 1 μM NS-398 for 3 h and treated with or without EP2A, EP4A or PGE_2 (100 nM each) for 4 h under serum-deprived conditions. (E) The number of mineralized nodules. Cells were chronically treated with or without EP2A, EP4A (100 nM each) or rBMP (10 ng/ml) alone, or various combinations of two of each reagent for 14 days. *p<0.05 and **p<0.01, compared to control (–). $^{\#}p$<0.05 and $^{\#\#}p$<0.01 ((B) compared to EP2A). N.S., no significant difference.

Figure 5. PGE_2 stimulates osteoblastogenesis through multiple MAPK pathways involved in PKA and PKC signaling.

(A) Cells were treated with or without EP2A or EP4A (100 nM each) for 5 and 30 min under serum-deprived conditions. (B) Cells were treated with or without EP2A (100 nM) or rNoggin (1 μg/ml) for 5 min under serum-deprived conditions. (C) The number of mineralized nodules. Cells were chronically treated with or without PGE_2 alone (100 nM) or in combination with MAPK inhibitors (10 μM each) for 14 days. (D) Real-time RT-PCR analysis of c-fos and Runx2. Cells at day 7 were pretreated with the MAPK inhibitors (10 μM each) for 3 h, followed by treatment with 100 nM PGE_2 for 30 min under serum-derived conditions. (E) Cells at day 7 were pretreated with or without 10 μM H89 or 1 μM Go6850 (Go) for 1 h, followed by treatment with 100 nM PGE_2 for 5 min under serum-deprived conditions. (F) Cells at day 7 were treated with 10 μM forskolin (FSK) or 1 μM PMA for 5 min. Western blot analysis was performed to determine activation of

MAPKs. P-p38, p-JNK and p-ERK indicate phosphorylated p38 MAPK (p38), JNK and ERK, respectively. SB, SB203580; D, dicumarol and U, U0126. *p<0.05 and **p<0.01 ((D), compared to PGE_2 alone).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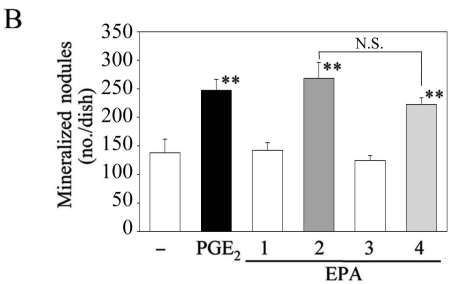
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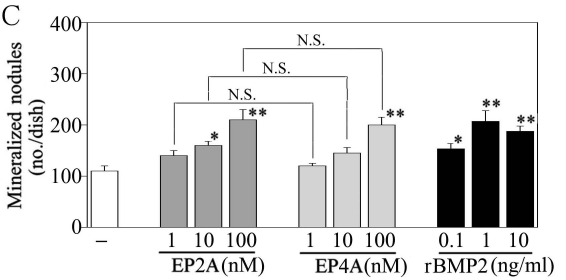
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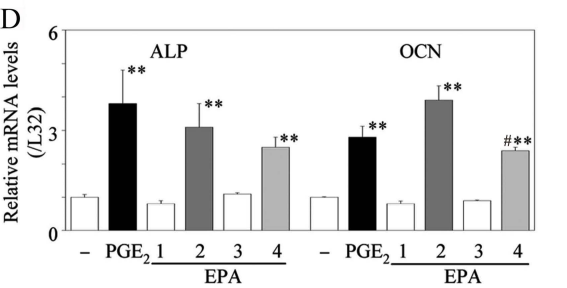
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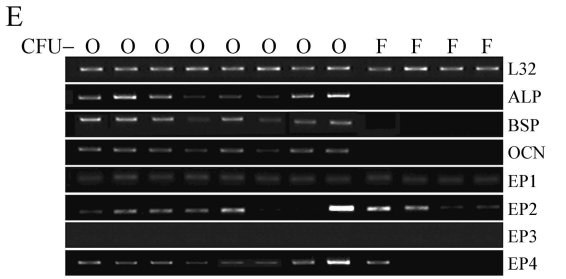
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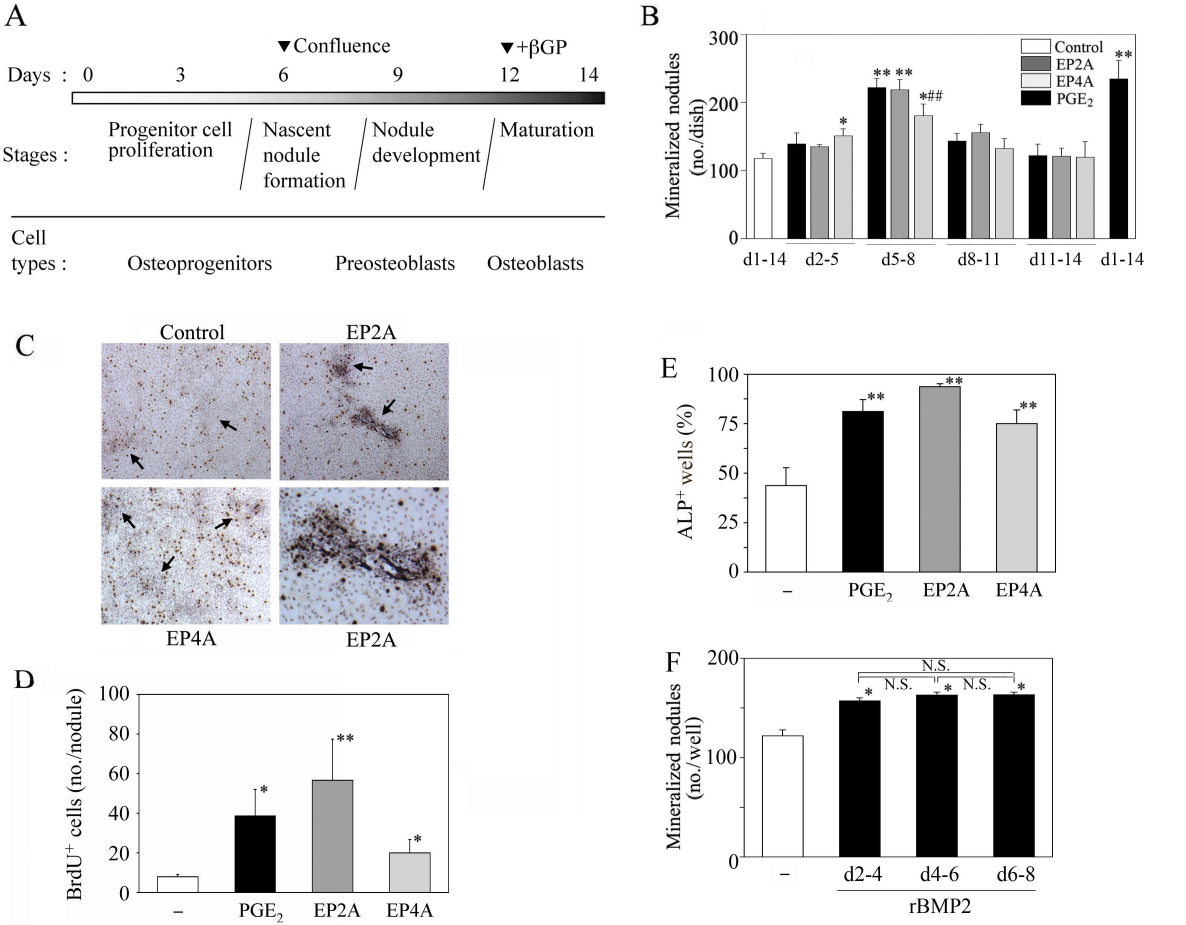
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Cell types :
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Preosteoblasts
Osteoblasts
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Mineralized nodules (no./dish)
Control
EP2A
EP4A
PGE2
d1-14
d2-5
d5-8
d8-11
d11-14
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C
Control
EP2A
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EP2A
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BrdU+ cells (no./nodule)
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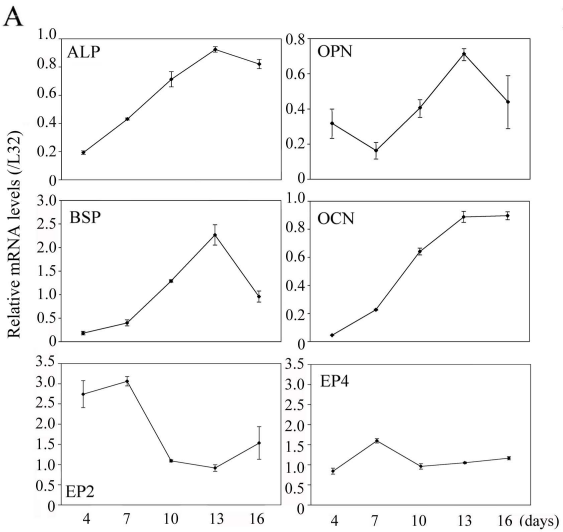
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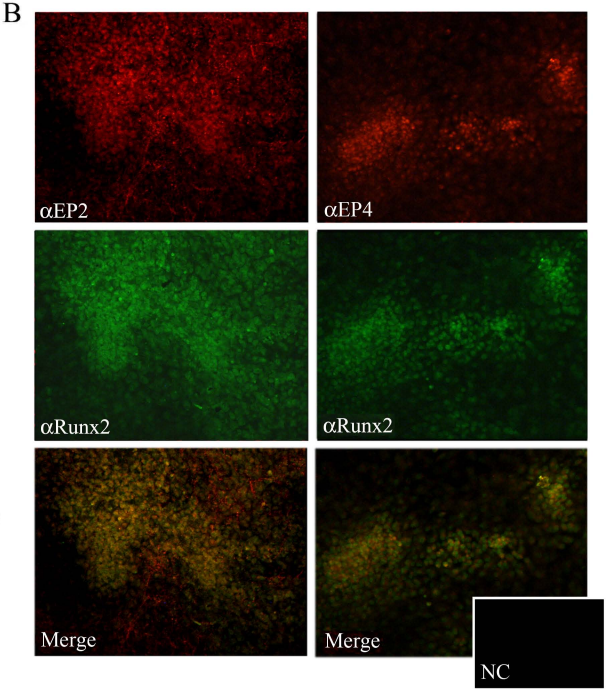
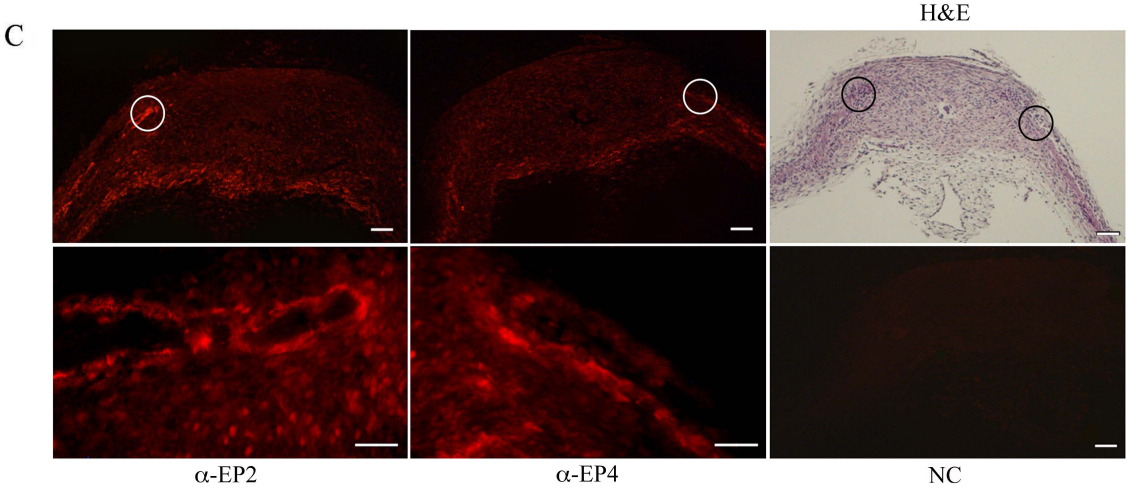
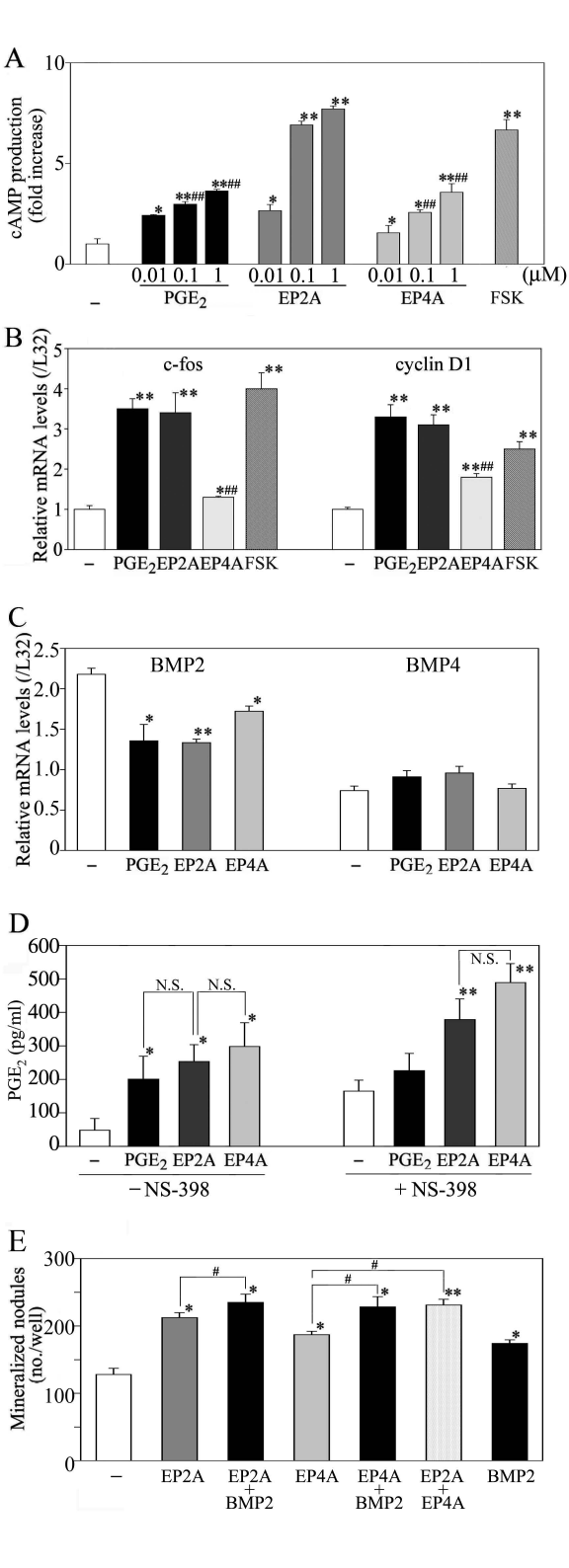
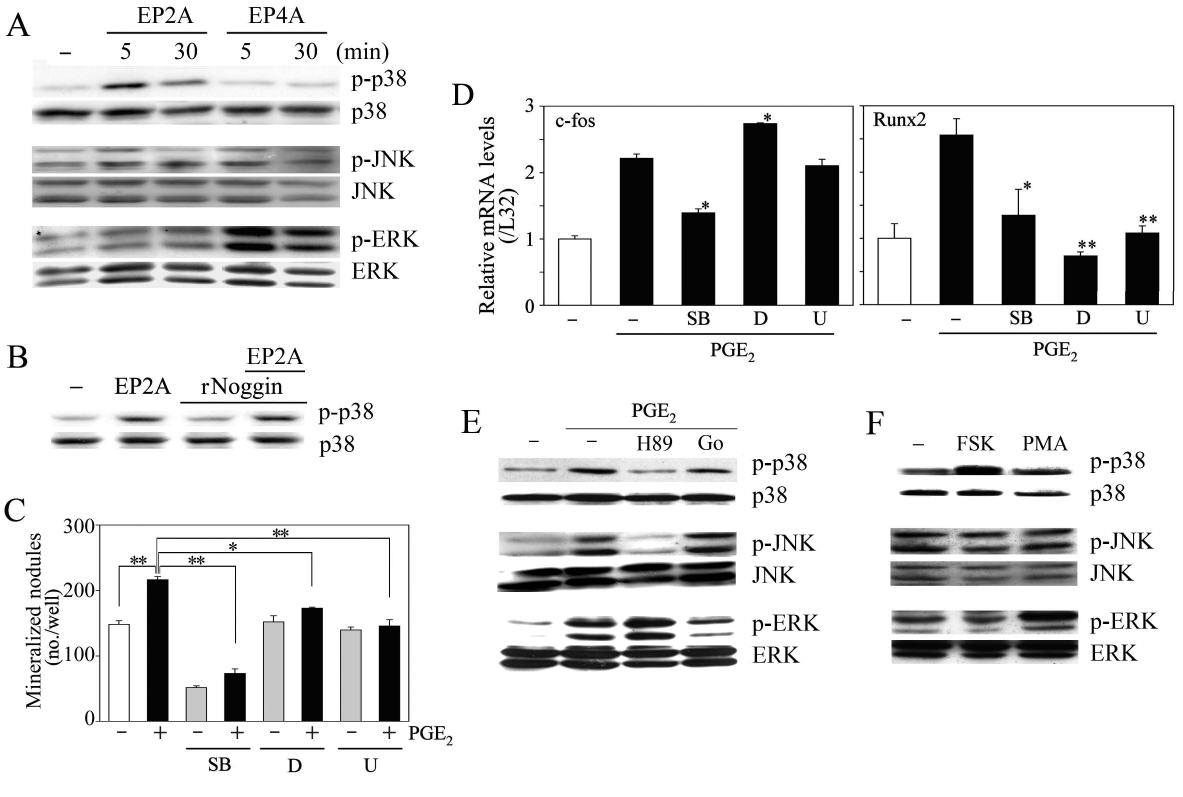
Relative mRNA levels (/L32)
4 7 10 13 16 (days)


B
αEP2
αEP4
αRunx2
αRunx2
Merge
Merge
NC


C
H&E
α-EP2
α-EP4
NC


A
cAMP production (fold increase)
10
5
0
0.01 0.1 1
0.01 0.1 1
0.01 0.1 1
(μM)
–
PGE2
EP2A
EP4A
FSK
B
Relative mRNA levels (/L32)
c-fos
cyclin D1
– PGE2 EP2A EP4A FSK
– PGE2 EP2A EP4A FSK
C
Relative mRNA levels (/L32)
BMP2
BMP4
– PGE2 EP2A EP4A
– PGE2 EP2A EP4A
D
PGE2 (pg/ml)
N.S.
N.S.
N.S.
– PGE2 EP2A EP4A
– PGE2 EP2A EP4A
−NS-398
+NS-398
E
Mineralized nodules (no./well)
– EP2A EP2A + BMP2 EP4A EP4A + BMP2 EP2A + EP4A BMP2


A
EP2A
EP4A
– 5 30 5 30 (min)
p-p38
p38
p-JNK
JNK
p-ERK
ERK
B
EP2A
– EP2A rNoggin
p-p38
p38
C
Mineralized nodules (no./well)
300
200
100
0
**
**
**
*
– + – + – + – + PGE2
SB D U
D
c-fos
Runx2
Relative mRNA levels (/L32)
3
2
1
0
*
*
**
**
– – SB D U
PGE2
E
PGE2
– – H89 Go
p-p38
p38
p-JNK
JNK
p-ERK
ERK
F
– FSK PMA
p-p38
p38
p-JNK
JNK
p-ERK
ERK